ORIGINAL ARTICLE

Expression of the cytolethal distending toxin in a geographically diverse collection of *Haemophilus ducreyi* clinical isolates

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Objective: To screen a collection of isolates of *Haemophilus ducreyi* for expression of the cytolethal distending toxin (CDT).
Methods: 45 clinical isolates of *H ducreyi* were screened for cytotoxic activity by examining the effect of culture supernatants on Hela cells. Expression was confirmed using immunoblotting with CDT specific monoclonal antibodies and the presence of the *cdt* genes determined by amplification of the *cdt* genes in a multiplex polymerase chain assay.
Results: Of the 45 clinical isolates, six isolates from differing geographical origins did not demonstrate cytotoxic activity. Expression of CDT was also not detected in these six isolates using immunoblotting and the genes *cdtA*, *cdtB*, and *cdtC* were not amplified using PCR. The remaining isolates demonstrated cytotoxic activity, expressed the CDT proteins, and the presence of the *cdt* genes was confirmed.
Conclusions: CDT is considered a virulence factor of *H ducreyi* but was found to be absent in 13% of isolates from different geographical origins.

Chancroid is a sexually transmitted infection (STI), primarily of the tropics, caused by the Gram negative bacterium *Haemophilus ducreyi*. The organism causes ulcers on the genitalia, often with inguinal lymphadenitis.[1] Untreated chancroid may persist for weeks or months, and can reoccur as there is no evidence of natural resistance, nor that past infection produces a protective immune response.[2 3] Genital ulcerative diseases have been shown in numerous studies to be strongly associated with the acquisition of HIV.[4]

Many virulence factors have been reported[1]; of particular interest is a cytolethal distending toxin (CDT), one of two toxins produced by the organism, the other toxin being a bacterial cell associated haemolysin.[5] The CDT is encoded by three closely linked genes—*cdtA*, *cdtB*, *cdtC*—which encode proteins with estimated molecular weights of 23 kDa, 29 kDa, and 19 kDa.[6 7] The majority of *H ducreyi* isolates contain this gene cluster,[8 9] indicating a significant role in pathogenesis.

The aim of this study was to screen a collection of *H ducreyi* isolates for cytotoxic activity, and to investigate the association with the *cdt* gene cluster.

MATERIALS AND METHODS

Strains of *H ducreyi*

Forty five clinical isolates of *H ducreyi* were used in this study (table 1), and were from genital ulcers, rather than buboes, in patients seen in diverse geographical locations. These isolates were a random selection, whose identity had been confirmed and were known to be of diverse ribotypes.[10 11] Two strains of *H ducreyi*, 35 000[12] and 512,[13] were used as positive and negative controls, respectively. *H ducreyi* CIP A77,[14] which does not produce lesions in the temperature dependent rabbit model,[15] was also included. Each isolate was retrieved from storage by inoculation onto a chocolate agar (CA) plate,[16] and incubated at 36°C in a humidified atmosphere with 5% carbon dioxide. The purity was confirmed by the presence of characteristic Gram negative coccibacilli, and the ability of the colonies to be pushed across the medium. They were then subsequently subcultured and 14 hour cultures were used in these experiments.

Preparation of culture supernatants

H ducreyi isolates were grown in a filter sterilised liquid medium.[16] After incubation, purity was checked and the broths centrifuged, then the supernatant was ultracentrifuged at 46 000 rpm for 90 minutes at 4°C, filter sterilised, and stored at −70°C.

Cytotoxic assays

HeLa B cells (ECACC 85060701) were seeded at 3×10^4 cells per well in a 24 well tissue culture plate.[17] After overnight incubation of the cells in 1 ml of tissue culture medium, an equal volume of filter sterilised bacteria culture supernatant was added to each well (three wells for each isolate), incubated for 3 hours before the fluid in each well was aspirated, and replaced with fresh tissue culture medium. The plates were incubated for 96 hours and then the test wells were compared with negative and positive control wells. Each well was scored as negative (confluent cells), positive (non-confluent cells), and photographed with an inverted phase contrast microscope. No graduations between positive and negative were noted after 96 hours of incubation.

Expression of CDT

Whole cells lysates were made from the pellets remaining after culture of *H ducreyi* strains in broth.[18] The proteins were resolved by SDS-PAGE on 15% gels,[19] with the outer two lanes loaded with rainbow markers (high molecular weight range, 14 300–220 000, and low molecular weight range, 2350–46 000, Amersham BioSciences). The proteins were transferred to nitrocellulose for western blot analysis[20] and then probed with the *H ducreyi* CdtA reactive Mab 1G8, CdtB reactive Mab 20B2, and CdtC reactive Mab 8C9 kindly provided by Dr E Hansen.[17] Anti-mouse IgG-HRP (Amersham Biosciences) was used to detect the antigen:antibody complex in each analysis and visualised using ECL detection reagents (Amersham Biosciences).

Amplification of *cdt* genes

Chromosomal DNA was prepared from cultures of each non-cytotoxin producing *H ducreyi* and a selection of cytotoxin

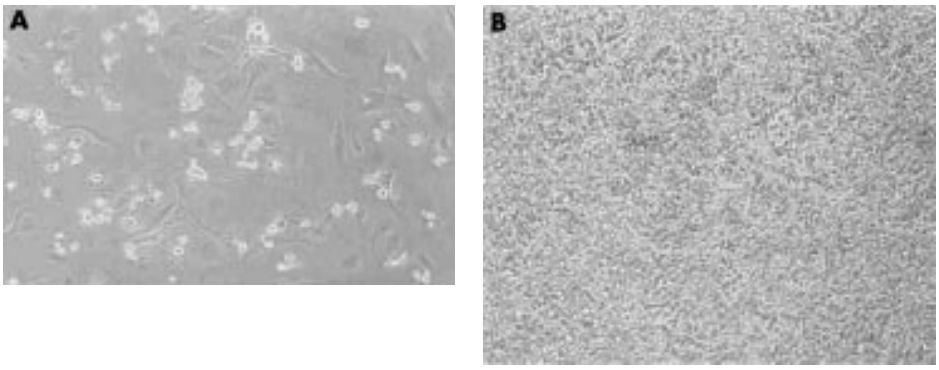

Figure 1 Effect of culture supernatant on a HeLa cell monlayer from (A) positive strain, D099 and (B) a negative strain, D033.

producing isolates harvested from chocolate agar plates incubated for 14 hours at 36°C (Qiamp kit, Qiagen).

A multiplex polymerase chain reaction (PCR) was constructed to determine the existence of any or all of the *cdt* genes. The sequences for each *cdt* primer pair were; *cdtA*, forward (cdt-5) 5′-^{128}AGG ATG GAT CTA AGG AGA G,^{146} 3′reverse (HB13–2) 5′-^{603}GTG CTA ATT TGT CCA GAC^{620}-3′(PCR product 475 bp); *cdtB*, forward (cdtBC) 5′-^{1179} GCA AAC CGA GTG AAC TTA G^{1198}-3′ reverse (cdt-1) 5′-^{1481}TAT TTT CAC TCA CTG CGG^{1498}-3′ (PCR product 320 bp); *cdtC*, forward (cdt-9) 5′-^{1984}ATG TTT TGC TTT CCT GGG^{2001}-3′ reverse (cdt-11) 5′-^{2237}ACC CTG ATT TCT TCG CAC^{2254}-3′ (PCR product 269 bp). Oligonucleotide primers to detect 16S ribosomal RNA of eubacteria were also added as a control, forward, 5′-^{509}AAC T(C/A)A CGT GCC AGC AGC CGC GGT A^{533}-3′ reverse 5′^{1517}-AAG GAG GTG ATC CA(G/A) CCG CA(G/C) (G/C)TT C^{1541}-3′.

The PCR mix used consisted of 5 µl of 10X PCR buffer (Gibco BRL), 3 µl 50 mM $MgCl_2$ (Gibco BRL), 2 µl 10 mM dNTPs (Boehringer Mannheim), 1 µl of each *cdt* primers (Sigma-Genosys) at a concentration of 50 pmol/µl, 2 µl of the 16S ribosomal RNA primers (Sigma-Genosys) at a concentration of 50 pmol per µl, 28 µl of dH_2O, 2 µl of target DNA, and 2 units *Taq* DNA polymerase (Gibco BRL). The PCR was performed in Robocycler Gradient 40 temperature cycler (Strategene), and the mix incubated at 94°C for 5 minutes to activate the *Taq* polymerase, followed by 25 cycles of 94°C for 1 minute, 51°C for 1 minute, and 72°C for 3 minutes. The final cycle was 72°C extension step for 5 minutes. The resulting PCR products were analysed using DNA 7500 chips on an Agilent bioanalyser (Agilent Technologies).

RESULTS

Cytotoxic assay results

In comparison with the control wells bacterial culture supernatants from 39 (87%) of the isolates, as exemplified by *H ducreyi* isolate D099 (fig 1A), exhibited HeLa cell cytotoxicity (table 1). After 96 hours, marked distension can be seen in the HeLa cells, continuing to the point where they disintegrate. At this time the cells detached from the substratum, destroying the cell monolayer. Supernatants obtained from six clinical isolates, including *H ducreyi* D033 (fig 1B), produced no cytotoxic activity. The monolayer of HeLa cells was not destroyed, but was intact growing across the base of the well to become confluent (table 1). *H ducreyi* CIP A77, known to be virulent in the animal model, also exhibited cytotoxicity.

Expression of CDT

In all isolates positive in the cytotoxic assay, a band corresponding to the putative form of CdtA of molecular mass of 23 kDa was detected (fig 2A). It was not present in isolates which were negative in the cytotoxic assay. Similar results were found for CdtB (fig 2B) and CdtC (fig 2C) with molecular masses of 29 kDa and 19 kDA respectively.

cdt genes

Amplification of *cdt* genes and control 16S rRNA in a multiplex PCR showed that isolates that were positive in the cytotoxic assay and expressed the protein each showed the expected PCR products of 475 bp (*cdtA*), 320 bp (*cdtB*), and 269 bp (*cdtC*)

Table 1 Cytotoxic activity of clinical isolates from various geographical locations

Strain	Strain No	Origin	Cytotoxic activity
35000	+ve control strain	Canada	+
512	–ve control strain	Thailand	–
A77	CIP A77	Institute of Pasteur	+
D001	KC	UK	+
D002	2087	S Africa	+
D003	V1157	USA	+
D005	3282	Kenya	+
D006	H80096	UK	+
D012	3144	Kenya	–
D013	3138	Kenya	+
D016	GU66	Gambia	+
D019	3216	Thailand	+
D023	E86934	UK	+
D026	36-F-2	France	+
D027	E96876	UK	–
D028	GU42	Gambia	+
D031	GU6	Gambia	+
D032	GU76	Gambia	+
D033	SHEFFD50	Sheffield	–
D034	V1159	USA	+
D037	GU73	Gambia	+
D038	GU69	Gambia	+
D039	3230	Thailand	–
D040	2684	Kenya	–
D041	SHEFFD1	Sheffield	–
D042	NCT10945	Vietnam	+
D043	P134	S Africa	+
D044	P117	S Africa	+
D045	P122	S Africa	+
D046	P137	S Africa	+
D047	P175	S Africa	+
D048	P136	S Africa	+
D049	P178	S Africa	+
D051	P138	S Africa	+
D053	P188	S Africa	+
D055	P172	S Africa	+
D056	P181	S Africa	+
D057	P189	S Africa	+
D061	P78	S Africa	+
D064	P153	S Africa	+
D065	P190	S Africa	+
D073	59162	Bangladesh	+
D076	ZAROWSKI	Antwerp	+
D080	P50	S Africa	+
D097	P70	S Africa	+
D099	P13	S Africa	+
D110	P19	S Africa	+
D111	P12	S Africa	+

Present = +, absent = –.

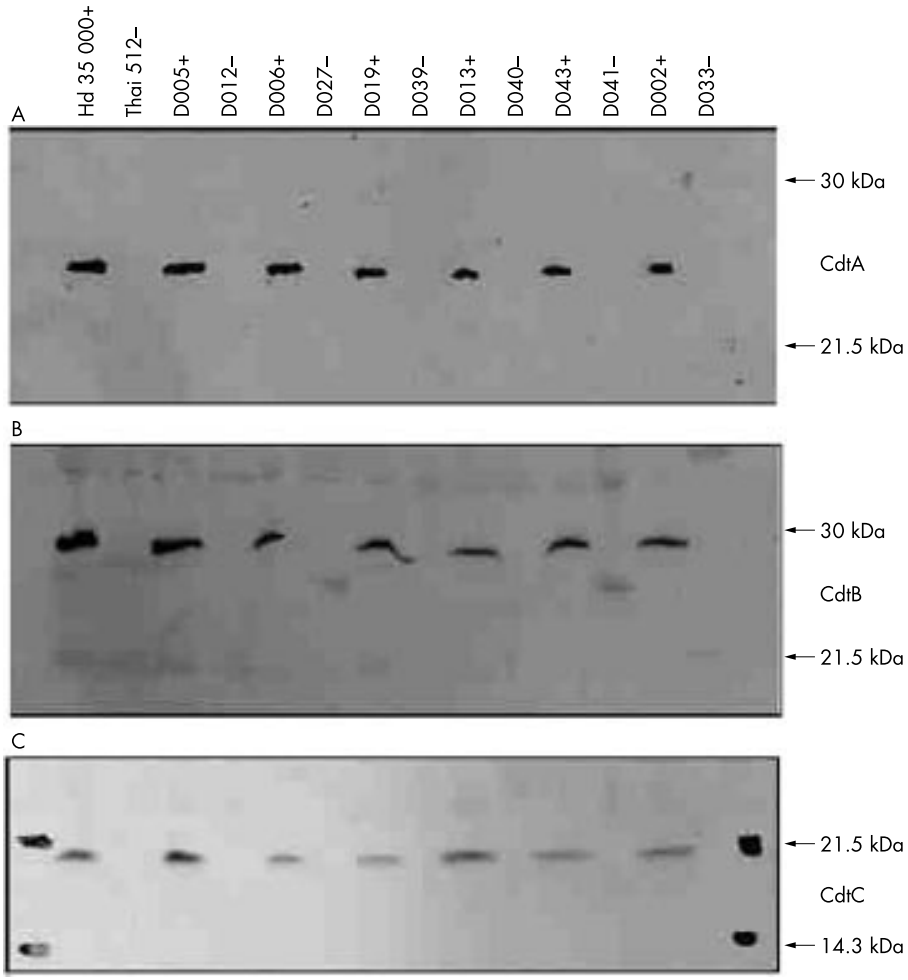

Figure 2 Expression of the proteins (A) CdtA, (B) CdtB, and (C) CdtC by isolates exhibiting no cytotoxic activty (–) and a selection of isolates showing cytotoxic activity (+). Strain 35 000 is the positive control and strain 512 is the negative control, D denotes a clinical isolate.

(fig 3). In each of the isolates that were negative in the cytotoxic assay and did not express the protein, only the 16S rRNA was amplified (fig 3), implying that these strains do not contain the *cdt* gene cluster.

DISCUSSION

In this study six out of the 45 isolates tested for cytotoxic activity were unable to induce cell death in HeLa cells, did not express any of the Cdt proteins, and the *cdt* gene cluster was not detected. The presence of the *cdt* gene cluster in 87% of isolates is similar to findings in other collections of isolates of 89%[8] and 82%.[9] The isolates in this study were isolated in different geographical locations but it is not possible to determine any particular association with country of isolation or source of infection because this was a random collection of isolates rather than a sentinel study.

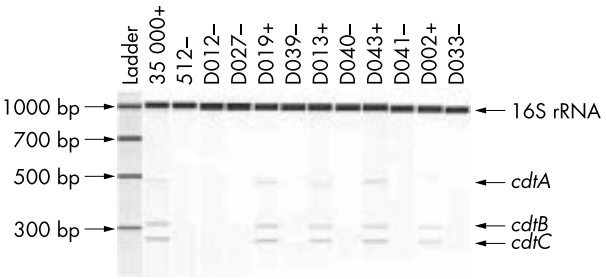

Figure 3 Amplification of the *cdtA*, *cdtB*, and *cdtC* genes in isolates exhibiting no cytotoxic activty (–) and a selection of isolates showing cytotoxic activity (+). Strain 35 000 is the positive control and strain 512 is the negative control, D denotes a clinical isolate.

After the original discovery of CDT by Johnson and Lior,[21] Purvén *et al*[8] described the subsequent characterisation of CDT effects in in vitro cell culture, and the soluble nature of *H ducreyi* CDT. In other bacterial species cytotoxic activity is known to be due to the expression of *cdtABC* gene cluster,[22–24] which encodes the cytolethal distending toxin. The effects of this toxin are not to be confused with that of the *H ducreyi* haemolysin, which is lethal for human foreskin fibroblasts (HFFs) but does not affect epithelial cells (HeLa cells),[25 26] whereas CDT is cytotoxic to many different cell lines.[6 27–29] A haemolysin deficient[30] CDT deficient mutant and a mutant deficient in both haemolysin and CDT[31] have been tested in the human model for experimental chancroid and have no effect on pustule formation, indicating neither has a direct role at this stage of the infection.

The role of CDT and its purpose in the course of chancroid remains unclear. It is known that CDT causes sensitive eukaryotic cells to be halted in the G2 phase of the cell cycle.[27] These cells, unable to proliferate, distend up to five times their original size before disintegrating (fig 1). The same phenotypic effects on the cell cycle have been detected in various studies with HIV infected cells, or cells transfected with *vpr*, an HIV regulatory gene.[32 33] It has been suggested that the CDT holotoxin has intrinsic DNase activity that is associated with the CdtB polypeptide.[34] It may be this DNase activity that is responsible for the cell cycle arrest, which CDT is capable of inducing.

In this study approximately 13% of the strains were non-cytotoxin producing, it is unlikely that these have arisen

spontaneously. It may be that *H ducreyi* originally did not possess the ability to produce CDT and the *cdt* gene sequence may have been acquired from a heterologous species. This is supported by the presence of transposase relics and one apparently intact transposase gene within 3 kb on either side of the *H ducreyi cdt* genes.[7]

CDT may be involved in genital ulceration in different ways. In the epidermis, it is able to cause the irreversible block of cell proliferation, consistent with the generation and slow healing of ulcers. After progressing through the epidermis, the toxin could act upon the fibroblasts in the dermis. *H ducreyi* CDT can destroy both keratinocytes and T lymphocytes,[35] suggesting that CDT may interfere with T cell responses to *H ducreyi* by apoptosis thus creating an environment that supports continued growth of the bacterium and facilitates enlargement and/or persistence of the ulcer. In this way it can be seen that *H ducreyi* CDT has potential to be a major virulence factor for the organism. Despite their in vitro effects these non-cytotoxin producing clinical isolates have been isolated from symptomatic patients, confirming the multivirulence determinant nature of the organism. The effect of haemolysin, the immune status of the individual and co-infection of other STIs, and the presence of a foreskin may all contribute to the ulceration in these patients, thereby compensating for the lack of CDT production.

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Conflicts of interest: none.

CONTRIBUTORS

KK undertook this study as part of an intercalated BSc in infection and immunity at the Faculty of Medicine, Imperial College of Science, Technology and Medicine and was awarded the MSSVD Undergraduate Science prize; KK was responsible for the technical work and preparation of the manuscript; DAL and CAI acted as supervisors and provided advice and critical review of the manuscript.

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